



TITLE:

OVOL2 Maintains the Transcriptional Program of Human Corneal Epithelium by Suppressing Epithelial-to-Mesenchymal Transition.

AUTHOR(S):

Kitazawa, Koji; Hikichi, Takafusa; Nakamura, Takahiro; Mitsunaga, Kanae; Tanaka, Azusa; Nakamura, Masahiro; Yamakawa, Tatsuya; ... Ueno, Morio; Kinoshita, Shigeru; Masui, Shinji

CITATION:

Kitazawa, Koji ...[et al]. OVOL2 Maintains the Transcriptional Program of Human Corneal Epithelium by Suppressing Epithelial-to-Mesenchymal Transition.. Cell reports 2016, 15(6): 1359-1368

ISSUE DATE:

2016-04-28

URL:

<http://hdl.handle.net/2433/210501>

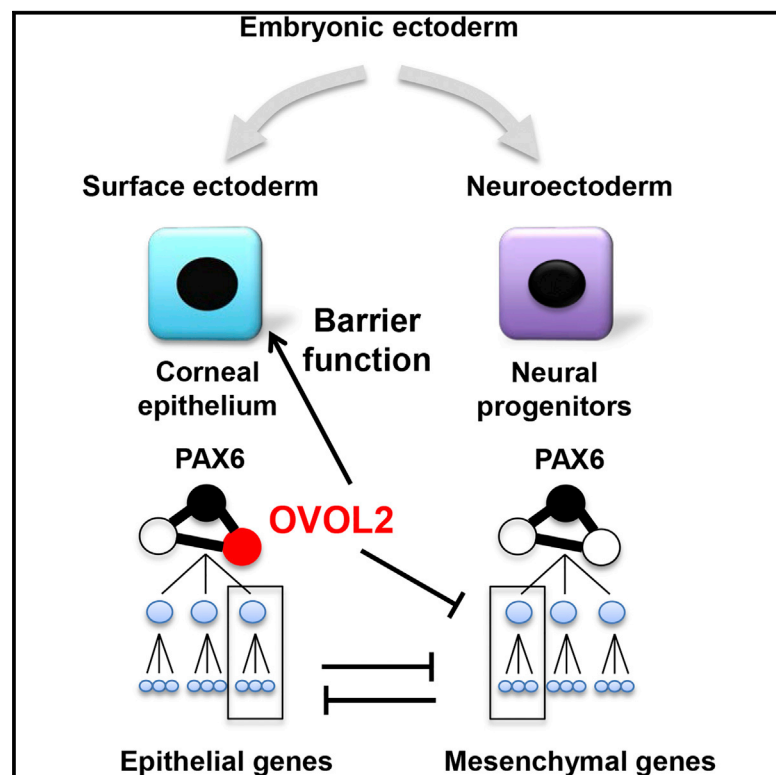
RIGHT:

© 2016 The Authors. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Cell Reports

OVOL2 Maintains the Transcriptional Program of Human Corneal Epithelium by Suppressing Epithelial-to-Mesenchymal Transition

Graphical Abstract



Authors

Koji Kitazawa, Takafusa Hikichi, Takahiro Nakamura, ..., Morio Ueno, Shigeru Kinoshita, Shinji Masui

Correspondence

shigeruk@koto.kpu-m.ac.jp (S.K.), smasui@cira.kyoto-u.ac.jp (S.M.)

In Brief

Kitazawa et al. show that the transcription factor OVOL2 maintains the transcriptional program of corneal epithelial cells by repressing mesenchymal genes and EMT. This may allow OVOL2 to regulate the differential transcriptional programs of the surface ectoderm and the neuroectoderm.

Highlights

- Identification of a transcription factor that can functionally maintain human CECs
- OVOL2 can activate CEC-specific genes in heterologous cell types
- OVOL2 represses mesenchymal genes in CECs
- EMT may regulate transcriptional programs of surface and neuroectoderm

Accession Numbers

GSE67823

Cell Reports Article

OVOL2 Maintains the Transcriptional Program of Human Corneal Epithelium by Suppressing Epithelial-to-Mesenchymal Transition

Koji Kitazawa,^{1,2,3,7} Takafusa Hikichi,¹ Takahiro Nakamura,^{2,3} Kanae Mitsunaga,¹ Azusa Tanaka,¹ Masahiro Nakamura,¹ Tatsuya Yamakawa,¹ Shiori Furukawa,¹ Mieko Takasaka,⁴ Naoki Goshima,⁵ Akira Watanabe,¹ Keisuke Okita,¹ Satoshi Kawasaki,^{2,6} Morio Ueno,² Shigeru Kinoshita,^{2,3,*} and Shinji Masui^{1,7,*}

¹Center for iPS Cell Research and Application, Kyoto University, 53 Kawahara-cho Shogoin Sakyo-ku, Kyoto 606-8507, Japan

²Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajicho Hirokoji-agaru Kawaramachi-dori Kamigyo-ku, Kyoto 602-0841, Japan

³Department of Frontier Medical Science and Technology for Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajicho Hirokoji-agaru Kawaramachi-dori Kamigyo-ku, Kyoto 602-0841, Japan

⁴JBIC Research Institute, Japan Biological Informatics Consortium, TIME24 Building 10F 2-4-32 Aomi Koto-ku, Tokyo 135-8073, Japan

⁵Molecular Profiling Research Center for Drug Discovery, National Institute of Advanced Industrial Science and Technology, Waterfront Bio-IT Research Building, 2-4-7 Aomi Koto-ku, Tokyo 135-0064, Japan

⁶Department of Ophthalmology, Osaka University, 2-2 Yamadaoka Suita, Osaka 565-0871, Japan

⁷CREST (Core Research for Evolutional Science and Technology), JST (Japan Science and Technology Agency), Honcho 4-1-8 Kawaguchi, Saitama 332-0012, Japan

*Correspondence: shigeruk@koto.kpu-m.ac.jp (S.K.), smasui@cira.kyoto-u.ac.jp (S.M.)

<http://dx.doi.org/10.1016/j.celrep.2016.04.020>

SUMMARY

In development, embryonic ectoderm differentiates into neuroectoderm and surface ectoderm using poorly understood mechanisms. Here, we show that the transcription factor OVOL2 maintains the transcriptional program of human corneal epithelium cells (CECs), a derivative of the surface ectoderm, and that OVOL2 may regulate the differential transcriptional programs of the two lineages. A functional screen identified OVOL2 as a repressor of mesenchymal genes to maintain CECs. Transduction of OVOL2 with several other transcription factors induced the transcriptional program of CECs in fibroblasts. Moreover, neuroectoderm derivatives were found to express mesenchymal genes, and OVOL2 alone could induce the transcriptional program of CECs in neural progenitors by repressing these genes while activating epithelial genes. Our data suggest that the difference between the transcriptional programs of some neuroectoderm- and surface ectoderm-derivative cells may be regulated in part by a reciprocally repressive mechanism between epithelial and mesenchymal genes, as seen in epithelial-to-mesenchymal transition.

INTRODUCTION

In development, embryonic ectoderm differentiates into either neuroectoderm or a non-neural ectoderm, such as surface ectoderm (Ozair et al., 2013), which gives rise to epidermal tissues including the corneal epithelium (Ordonez and Di Girolamo,

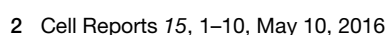
2012). However, the mechanism regulating specification between these lineages is largely unknown. Transcription factors (TFs) are crucial in lineage specification, as they regulate cell-type-specific transcriptional programs (Lee and Young, 2013). There are more than 2,000 TFs in the mammalian genome and hundreds are differentially expressed in each cell type. A handful of TFs, or master TFs, are able to regulate a whole transcriptional program (e.g., direct reprogramming) and play a central role in determining cellular identity (Lee and Young, 2013). The transcriptional program of neuroectoderm requires the paired box TF PAX6 (Zhang et al., 2010), which is also necessary for that of corneal epithelial cells (CECs) (Ouyang et al., 2014). The fact that these two closely related lineages require a common TF implies that a substantial part of their maintenance mechanisms is shared. TFs for neuroectodermal derivatives, such as neural progenitors, have been studied extensively (Xu et al., 2015) but those of human CECs are relatively unknown.

In the present study, we identified OVOL2 as a new TF that maintains CECs. OVOL2 functioned by mainly repressing mesenchymal genes. We also found that neuroectoderm lineage cells expressed mesenchymal genes, of which repression by OVOL2 and activation of epithelial genes could induce the transcriptional program of CECs. This process was likely regulated by a reciprocally repressing mechanism between epithelial and mesenchymal genes, as seen in epithelial-to-mesenchymal transition (EMT). These results should shed new light on the transcriptional programs determining neuroectoderm and surface ectoderm lineage.

RESULTS

TFs that Inhibit iPSC Induction in CECs Tend to Regulate Epithelial Development

Because TFs that strongly maintain cell-type-specific transcriptional programs are reported to inhibit the dedifferentiation



expression levels between primary CECs and fibroblasts (Figures S2C and S2D). Moreover, principle component analysis (PCA) revealed that 6TFs-fib were mapped closer to primary CECs and distant from fibroblasts (Figure S2E), mostly because of the expression of genes involved in development or differentiation (Tables S1 and S2). This indicates that these six TFs were able to at least partially induce the transcriptional program of CECs.

In addition to global gene expression, the landscape of nucleosome-free regions highly reflects differences in cell lineages, likely because lineage-specific TFs bind and regulate gene expressions through these regions (Stergachis et al., 2013). Accordingly, we performed assay for transposase-accessible chromatin with high throughput sequencing (ATAC-seq), which provides genome-wide information on nucleosome-free regions (Buenrostro et al., 2013), using CECs, 6TFs-fib, and fibroblasts. Notably, in some CEC-specific genes, peaks in the promoter regions seen in primary CECs but not in fibroblasts appeared in 6TFs-fib, whereas peaks in the promoter regions of some fibroblast-specific genes seen in fibroblasts disappeared in 6TFs-fib (Figures 2G and S2F). Global comparison of peaks indicated that 17.3% of peaks that appeared in 6TFs-fib (6TFs-fib presence peaks) were primary CEC-specific peaks, while 61.3% of peaks that disappeared (6TFs-fib absence peaks) were fibroblast-specific peaks (Figures 2H and S2G), indicating that the six TFs were able to at least partially induce a CEC landscape of nucleosome-free regions in fibroblasts.

OVOL2 Repressed Mesenchymal Genes in CECs

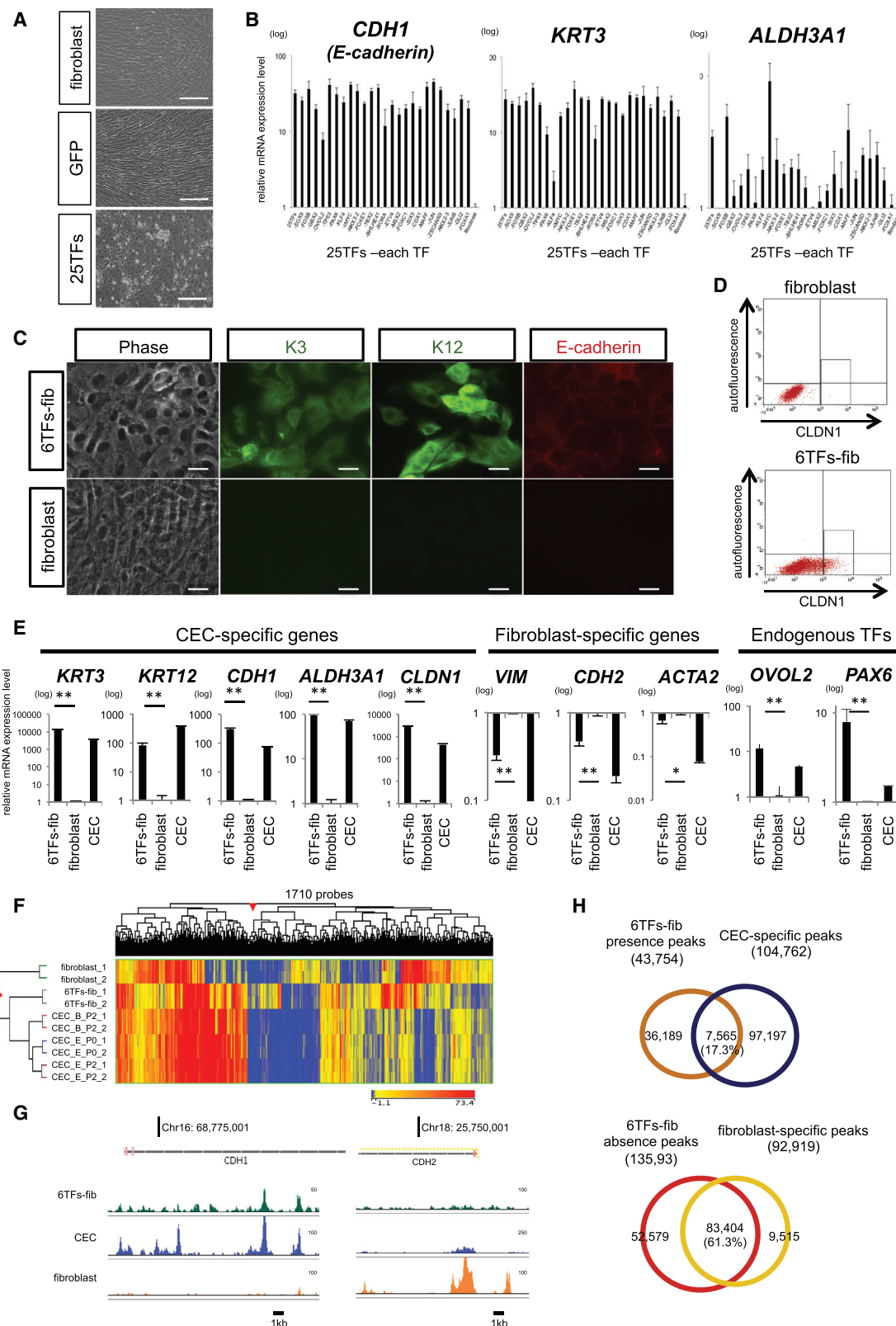
Because the functions of PAX6 and KLF4 in CEC maintenance have already been reported (Graw, 2003; Swamynathan et al., 2007), we next examined how OVOL2 contributes to maintaining the transcriptional program of CECs using small interfering RNA (siRNA) in primary CECs. OVOL2 knockdown reduced the expression of CEC-specific genes (Figure 3A) and keratin 12 protein in primary CECs (Figure 3B). Morphologically, CECs with OVOL2 knockdown became elongated fibroblastic cells (Figure 3C). Immunocytochemistry of CECs with OVOL2 knockdown showed a reduction of K12 and E-cadherin proteins (Figure 3D). Moreover, OVOL2 knockdown significantly reduced the barrier function of primary CECs as measured by transepithelial electrical resistance (TER) (Figures 3E and 3F), suggesting that OVOL2 functionally maintains CECs. Global expression analyses between CECs and CECs treated with siOVOL2 by microarray revealed that among the 42,545 genes analyzed, 805 genes were differentially expressed by >1.5-fold, and 320 genes were upregulated and 485 genes were downregulated in CECs with siOVOL2 (Figure 3G). Downregulated genes enriched GO terms related to epithelial functions (Figure 3H), whereas upregulated genes were not significantly associated with any terms.

To gain insight into the function of OVOL2, we analyzed TF motif enrichment using the ATAC-seq data. In sequences among the fibroblast-specific peaks and the 6TFs-fib absence peaks, the OVOL2 motif (CCGTTA) (Watanabe et al., 2014) was significantly enriched (Figure S3A; Table S3A). Moreover, genes within the 6TFs-fib absence peaks and containing the OVOL2 motif enriched GO terms related to mesenchymal cells (Figure S3B). To address whether OVOL2 represses mesenchymal genes in

CECs, we examined OVOL2 effects in response to TGF- β , whose activation leads to a loss of CEC identity through EMT (Yamben et al., 2013). We used the doxycycline (Dox)-inducible system to conditionally overexpress OVOL2 in primary CECs. While CECs treated with TGF- β changed to fibroblastic morphology (Figure S3C) and showed an upregulation of mesenchymal genes (Figure S3D), OVOL2 overexpression at least partially blocked the morphological change and significantly repressed the upregulation (Figures S3C and S3D), indicating that OVOL2 represses mesenchymal genes in CECs. Indeed, in OVOL2 knockdown CECs, mesenchymal genes such as *ZEB1* and *ZEB2*, two well-known mesenchymal genes (De Craene and Berx, 2013), were upregulated (Figure S3E). Chromatin immunoprecipitation sequencing (ChIP-seq) analysis using CECs showed OVOL2 peaks on *ZEB1* and *ZEB2* (Figure 3I; Table S3B), suggesting that OVOL2 bound and repressed fibroblast-specific genes, particularly those with mesenchymal function. In accordance with this result, recent reports showed that OVOL2 is a strong epithelial regulator that maintains transcriptional programs in epidermal keratinocytes and mammary epithelial cells by repressing *ZEB1* and *ZEB2* (Lee et al., 2014; Watanabe et al., 2014). At the same time, overexpression of *ZEB1* in CECs partially repressed the expression level of *CDH1* (Figure 3J), consistent with its effect in other epithelial cells (Shirakihara et al., 2007), and E-cadherin expression was weak in CECs overexpressing *ZEB1* or *ZEB2* compared to the control (Figure 3K). Additionally, barrier function assay demonstrated that CECs overexpressing *ZEB1* or *ZEB2* reduced the TER compared to the control (Figure 3L). These results suggested that OVOL2 maintains CECs in part by repressing mesenchymal genes such as *ZEB* genes.

OVOL2 Induced the Transcriptional Program of CECs in Neural Progenitor Cells

Interestingly, OVOL2 knockdown in CECs derepressed not only mesenchymal genes such as *ZEB* genes, but also neural progenitor cell (NPC)-specific genes including *SOX2* (Figure S3E). *ZEB* genes are required for neuroectoderm differentiation in vivo and in vitro (Chng et al., 2010; Miyoshi et al., 2006). We meta-analyzed published datasets of human cells and found that in neuroectoderm and NPCs, some mesenchymal genes including *ZEB* genes are highly expressed, whereas epithelial genes are repressed (Figure 4A). This expression pattern was similar to that of adipose-derived mesenchymal stem cells and fibroblasts (Figure 4A), which are known to express mesenchymal genes (Kfoury and Scadden, 2015). In contrast, in CECs and epidermal keratinocytes, which are derived from surface ectoderm (Koster and Roop, 2007), as well as in iPSCs and ESCs, which are thought to be epithelial cells (De Craene and Berx, 2013), the expression pattern was opposite; mesenchymal genes were repressed whereas epithelial genes were highly expressed (Figure 4A). We further meta-analyzed additional datasets of mouse cells and confirmed similar expression patterns (higher expression of mesenchymal genes; lower expression of epithelial genes) in various cell types derived from neuroectoderm (Figures S4A and S4B). These results suggest that mesenchymal genes might contribute to the maintenance of transcriptional programs of neuroectoderm lineage cells, and epithelial genes



(legend on next page)

expressed in surface ectodermal lineage cells possibly repress the transcriptional program of neuroectoderm cells by repressing mesenchymal genes using a mechanism similar to that in EMT (De Craene and Berx, 2013). We thus hypothesized that OVOL2 regulates cell-type-specific transcriptional programs between CECs and NPCs, which we used as representative cell types of surface ectoderm and neuroectoderm lineage, respectively, through the reciprocally repressive mechanism between epithelial and mesenchymal genes seen in EMT. To test this theory, we overexpressed OVOL2 alone in NPCs and analyzed the expression of CEC-specific genes. To our surprise, NPCs with OVOL2 overexpression started to form cell-cell junctions much like those in epithelial-like cells (Figure 4B). NPCs with OVOL2 overexpression exhibited a high expression of CEC-specific genes (*Krt12* and *Aldh3a1*) without any upregulation of *Krt1* or *Krt10*, while mesenchymal genes (*Snai2* and *Zeb2*) were downregulated (Figures 4C and S4C). Furthermore, by microarray analysis, we found that the expressions of most epithelial and mesenchymal genes were reciprocated in NPCs with OVOL2 and NPCs alone, with epithelial genes upregulated and mesenchymal genes downregulated in the former (Figure 4D). In light of the specificity of *Krt12* expression (as well as the phenotype of its knockout mice) seen only in corneal epithelium in the body (Kao et al., 1996), the upregulation of *Krt12* by OVOL2 overexpression suggests specific activation of the transcriptional program of CECs. We considered whether this activation was mediated, in part, by the repression of *Zeb1* and *Zeb2*, because both genes were quickly downregulated in NPC overexpressing OVOL2 (Figure 4E), while at the same time *Zeb1* overexpression partially blocked the upregulation of *Krt12* and *Cdh1* (Figure 4F). These results are consistent with our ChIP-seq data (Figure 3I) and demonstrate that ZEB1/2 were OVOL2 targets. At the global level, the upregulated and downregulated genes contained a significant number of genes highly expressed in CECs and NPCs, respectively (Figures S4D and S4E). These data indicated that forced expression of OVOL2 in NPCs could at least partially induce the transcriptional program of CECs by repressing mesenchymal genes. Moreover, overexpression of *Grlh2*, another epithelial TF (De Craene and Berx, 2013), induced epithelial-like cells in NPCs (Figure S4F) with an expression profile similar to that by OVOL2 overexpression (Figure S4G), suggesting the repression of mesenchymal genes and activation of epithelial genes (i.e., regulation of EMT-related genes) may be sufficient to activate the transcriptional program of CECs. Collectively, our data show that OVOL2 maintains the transcriptional program of CECs by repressing mesenchymal genes to maintain epithelial identity (Figure 4G). This result suggests that

differences between the transcriptional programs of some surface ectoderm- and neuroectoderm-derivative cells may be regulated in part by the reciprocally repressive mechanism between epithelial and mesenchymal genes seen in EMT.

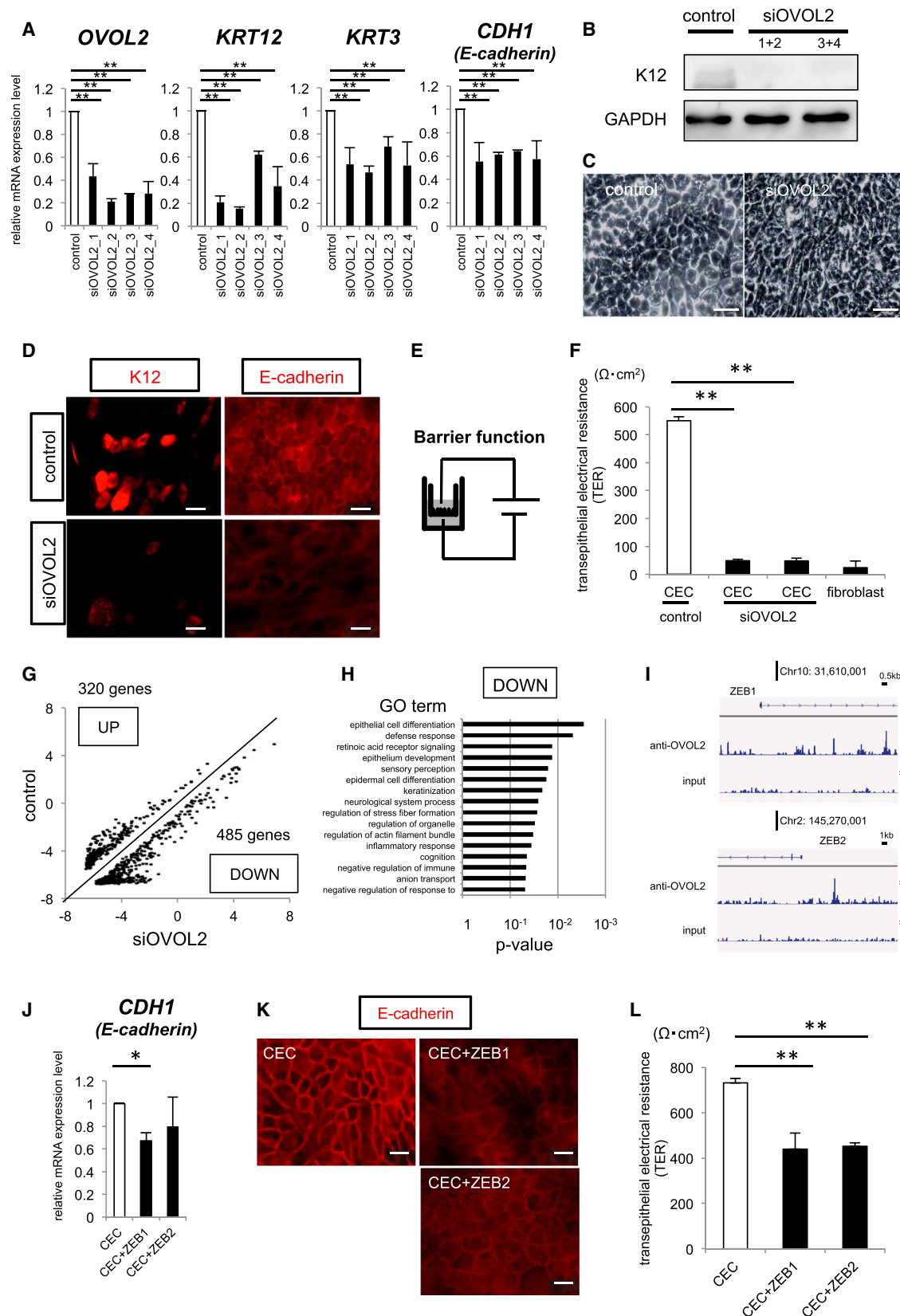
DISCUSSION

The identification of master TFs has advanced our understanding of development and aided in therapeutic research (Lee and Young, 2013). Previously, PAX6 was the only identified TF that could induce CEC-lineage cells from a derivative of surface ectoderm (Ouyang et al., 2014). In the present work, we found PAX6 alone did not induce the transcriptional program of CECs in fibroblasts, whose lineage is presumably distant from surface ectoderm (Kfoury and Scadden, 2015) (Figure S2A). Instead, cotransduction with OVOL2, an epithelial regulator, successfully induced the transcriptional program. Overexpression of OVOL2 alone in fibroblasts, however, was not sufficient for the induction (Figure S2A) suggesting that PAX6 and OVOL2 may cooperatively activate the transcriptional program of CECs. Indeed, overexpression of OVOL2 with other TFs could induce CEC-like cells in a cell type (oral mucosal epithelial cell) other than fibroblasts (Figure S4H), suggesting that OVOL2 could provide important clues for future therapeutics.

Our data showed that OVOL2 maintained the transcriptional program of CECs by repressing mesenchymal genes, a function that may explain differences in the transcriptional programs of neuroectoderm and surface ectoderm. Several lines of evidence support this scheme. In *Ovol2* knockout embryo, surface ectoderm is reduced while neuroectoderm is expanded (Mackay et al., 2006). In addition, in an ESC differentiation system, *Ovol2* knockdown enhances neuroectoderm differentiation, and *Ovol2* overexpression in chick embryo inhibits neuroectoderm differentiation (Mackay et al., 2006; Zhang et al., 2013). On the other hand, mesenchymal genes are required for the transcriptional program of neuroectoderm, as knockout of *ZEB1* and *ZEB2* in embryo causes defects in neural tube in which *Sox2* (a TF gene required for neuroectoderm) expression is repressed (Miyoshi et al., 2006), and *ZEB2* knockdown in ESCs suppresses neuroectoderm differentiation (Chng et al., 2010). Several other studies using ESCs have also indicated the involvement of mesenchymal genes in neuroectoderm differentiation (Di Micco et al., 2014; Du et al., 2013; Gill et al., 2011). Moreover, our analysis showed that various neuroectoderm derivatives expressed high levels of mesenchymal genes, while surface ectoderm derivatives instead expressed epithelial genes highly. These observations suggest that mesenchymal and epithelial genes, which might segregate

Figure 2. Combined Overexpression of Inhibitory TFs Induced the Transcriptional Program of CECs in Human Fibroblasts

- (A) The morphology of neonatal human fibroblasts, fibroblasts transduced with GFP, and fibroblasts transduced with 25 TFs. Scale bars, 200 μ m.
(B) The effect of removing individual TFs from the pool of 25 TFs was examined by qRT-PCR (all $n = 4$). Data are shown as means \pm SD.
(C) Phase contrast and immunocytochemistry of K3, K12, and E-cadherin in fibroblasts and fibroblasts with 6 TFs (6TFs-fib). Scale bars, 20 μ m.
(D) Fluorescence-activated cell sorting (FACS) analysis using Claudin 1 (CLDN1) antibody.
(E) Quantitative analysis of CEC-specific genes, fibroblast-specific genes, and endogenous *OVOL2* and *PAX6* in the indicated cells (all $n = 4$). Data were analyzed using the unpaired t test and are shown as means \pm SD. * $p < 0.05$, ** $p < 0.01$.
(F) Unsupervised hierarchical analyses of global gene expression.
(G) ATAC-seq peaks in the promoter regions of CEC-specific genes and fibroblastic-specific genes (all $n = 4$).
(H) Comparison of ATAC-seq peaks at the global level. Numbers represent peak numbers detected by Hotspot software.
See also Figure S2 and Table S2.



(legend on next page)

neuroectoderm and surface ectoderm in early development, may still regulate the transcriptional programs in some derivative cells of these lineages. Recent reports have demonstrated that OVOL2 maintains epidermal keratinocytes and mammary epithelial cells by repressing mesenchymal genes, suggesting it an epithelial regulator (Lee et al., 2014; Watanabe et al., 2014). Our data showed that in CECs, OVOL2 activates epithelial genes by repressing mesenchymal genes whose expression might promote neuroectoderm lineage. Similarly to other epithelial cell types, OVOL2 bound to and could repress *ZEB1* and *ZEB2* in CECs, suggesting that both of these genes are direct targets of OVOL2. Because upregulation of *ZEB1* and *ZEB2* was modest in OVOL2 knockdown, these two genes might constitute only a subgroup of causative genes for the loss of epithelial identity, as seen in keratinocytes (Lee et al., 2014). Taken together, we propose that OVOL2 might regulate differences in the transcriptional programs between surface ectoderm and neuroectoderm through a reciprocally repressing mechanism between epithelial and mesenchymal genes, as seen in the switch-like reversion of the expression status in EMT.

EXPERIMENTAL PROCEDURES

Sample Collection and Cell Preparation

Human normal corneal tissues were obtained from donors from SightLife exclusively for research purposes and human normal conjunctival tissues from two patients who received conjunctival-chalasis surgery. Human oral mucosal tissues were obtained from healthy volunteers, and superfluous tissue was obtained from patients undergoing oral surgery. All patients provided informed consent following the guidelines of the Kyoto Prefectural University of Medicine Ethics Committee. CECs were isolated by treatment with 1,000 protease units (PU)/ml of dispase (Dispase type II; Godo Shusei) at 4°C overnight and TrypLE Express (Life Technologies) at 4°C for 30 min. Isolated primary CECs were seeded on a 60-mm dish or 6-well plate coated with 0.1% gelatin and were cultured in CEC medium, which consisted of DMEM and Ham's F-12 media (1:1 mixture) (Life Technologies) containing B27-supplement (Life Technologies), Rho-kinase (ROCK) inhibitor (10 mM) (Nacalai Tesque), epidermal growth factor (EGF) (10 ng/ml) (Life Technologies), Epigallocatechin gallate (10 ng/ml) (Sigma), and penicillin-streptomycin (50 IU/ml) (Nacalai Tesque), at 37°C in a 5% CO₂ incubator and then used for the following experiments after one passage using TrypLE Express at double dilution at room temperature upon reaching sub-confluence. For the functional screening of transcription factors, CECs from the same donors at the time of

first passage were stocked at –150°C until use. Neonatal human dermal fibroblasts were purchased from Lonza. NPCs have been previously established and cultured in N2B27 media with fibroblast growth factor (FGF) 2 and EGF (Conti et al., 2005; Hikichi et al., 2013).

Retroviral Preparation

Plat-GP cells were seeded at a density of 2.0×10^6 cells on a 10-cm dish in 10 ml of DMEM (Nacalai Tesque) with 10% FBS. Retroviral (6 μg) and 3 μg of VSV-G vector DNAs were transfected by using 27 μl of FuGENE 6 (Promega). A mixture of the retroviral supernatant and the cell culture medium in equal amounts was added to the cell in the presence of 5 μg/ml polybrene on the plate. The cell plate was set up in the centrifuge at 35°C and then centrifuged for 30 min at 800 × g.

iPSC Induction Assay

Yamanaka factors (OCT3/4, SOX2, KLF4, and cMYC) with each candidate TF (total, 5 TFs) were transduced by retroviruses to the CECs seeded at a density of 0.75 to 1.0×10^4 cells on a 6-well plate. At day 2, mitomycin-treated SNL feeder cells were added at a density of 5×10^5 cells, and the medium was replaced with CEC medium and 0.5% FBS. After day 6, the medium was switched to Primate ES cell medium (ReproCELL) supplemented with 20 ng/ml FGF2 and changed each day until day 21. iPSC colonies were stained by alkaline phosphatase (AP) Kit (Muto Pure Chemicals).

Induction of the Transcriptional Program of CECs in Fibroblasts

A combination of TFs was transduced by lentivirus or retrovirus to fibroblasts seeded at a density of 6.0×10^4 cells on a 6-well plate. After 3 days of infection, the medium was switched to CEC medium, and the cells were cultured until day 10. RNA was extracted using the RNeasy Mini Kit. Primers listed in Table S4 were used for qRT-PCR.

siRNA Treatment

ON-TARGETplus siRNA or siRNA-SMARTpool (25 nM) (GE Healthcare) was transfected into the primary CECs at a density of 2.5×10^5 cells on a 24-well plate using 2.5 μl of DharmaFECT 4 siRNA Transfection Reagent (GE Healthcare). The cells were harvested at 48 hr after the transfection.

Barrier Functional Assay

Cells were cultured on 12-well porous membrane filters (Transwell, 0.4 mm pore; Corning) at 5.0×10^5 cells. TER between the upper and lower chambers was assessed using a volt-ohm meter (EVOM; World Precision Instruments) as previously described (Kitazawa et al., 2013).

Induction of the Transcriptional Program of CECs in NPCs

Retroviruses were transduced to NPCs at 5.0×10^3 cells on a 6-well plate, and the next day 1 μg/ml puromycin or 500 μg/ml neomycin was added. For the

Figure 3. OVOL2 Repressed Mesenchymal Genes in CECs

- (A) Knockdown of OVOL2 using siRNA (siOVOL2) in CECs and downregulation of CEC-specific genes based on qRT-PCR (all n = 4). Data were analyzed using the unpaired t test and are shown as means ± SD. **p < 0.01.
- (B) Western blotting of K12 in primary CECs treated with two siRNAs for OVOL2.
- (C) The morphology of CECs and CECs treated with siOVOL2. Scale bars, 20 μm.
- (D) Immunocytochemistry of K12 and E-cadherin in CECs and CECs treated with siOVOL2. Scale bars, 20 μm.
- (E) Barrier function was assessed by measuring transepithelial electrical resistance (TER) with a volt-ohm meter.
- (F) Barrier function assay demonstrated that siRNA for OVOL2 reduced the TER compared to control. Representative results of two independent experiments with technical triplicates are shown as means ± SD. Data were analyzed using the unpaired t test. **p < 0.01.
- (G) Scatter plots analysis of gene expressions between CECs and CECs treated with siOVOL2 found upregulated genes (320 genes) and downregulated genes (485 genes).
- (H) GO analysis showed that epithelial-related genes were enriched in DOWN genes.
- (I) ChIP-seq analysis of OVOL2 in the CECs.
- (J) Quantitative analysis of *CDH1* in the CECs and CECs overexpressing *ZEB1* or *ZEB2* (all n = 3). Data were analyzed using the unpaired t test and are shown as means ± SD. *p < 0.05. GFP-transduced CECs were used as control.
- (K) Immunocytochemistry of *CDH1* in CECs and CECs overexpressing *ZEB1* or *ZEB2*. GFP-transduced CECs were used as control.
- (L) Barrier function assay demonstrated that overexpression of *ZEB1* or *ZEB2* in CECs reduced the TER compared to the control (GFP-transduced CECs). Representative results of four independent experiments with technical triplicates are shown as means ± SD. Data were analyzed using the unpaired t test. **p < 0.01. See also Figure S3 and Table S3.

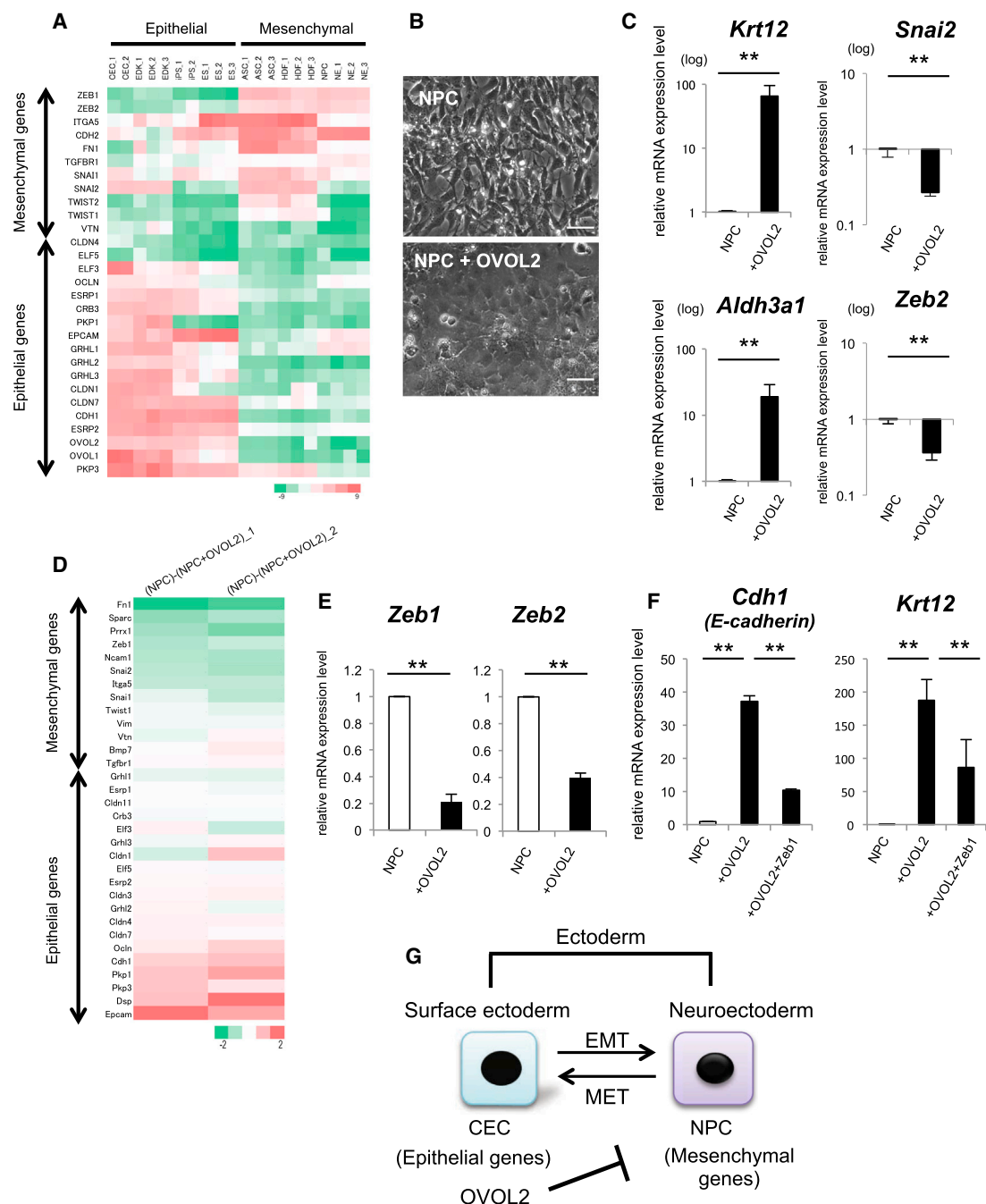


Figure 4. OVOL2 Overexpression Induced the Transcriptional Program of CECs in Neural Progenitor Cells by Regulating Genes Involved in EMT

(A) Microarray analyses of EMT-related genes among CECs, epidermal keratinocytes (EDK), adipose-derived stem cells (ASC), dermal fibroblasts (HDF), NPCs, neuroectoderm (NE), iPSCs, and ESCs.

(B–D) Ten days after viral infection. (B) The morphology of NPCs overexpressing OVOL2. Scale bars, 20 μ m. (C) Quantitative analysis of CEC-specific genes and NPC-specific genes (all $n = 4$). Data were analyzed using the unpaired t test and are shown as means \pm SD. ** $p < 0.01$. (D) The expressions of EMT-related genes reciprocate with the overexpression of OVOL2. Two individual experiments were performed.

(E and F) Three days after viral infection. Data were analyzed using the unpaired t test and are shown as means \pm SD. ** $p < 0.01$. (E) *Zeb1* and *Zeb2* were downregulated in NPC overexpressing OVOL2. (F) OVOL2 activated, but *Zeb1* repressed *Krt12* and *Cdh1* gene expressions in NPC.

(G) Schematic figure of the mechanism shows how the transcriptional profile between CEC and NPC is distinguished.

See also Figures S3 and S4.

samples of 10 days after transduction, at 3 days after transduction the culture medium was switched to N2B27 medium with EGF but without FGF and cultured 7 days.

Statistics

Unpaired t tests were used for all qRT-PCR analyses. For the binding motif analysis of OVOL2 between CECs and fibroblasts, Fisher's exact test was used. Pearson's correlation coefficient was used for comparisons of iPSC induction activity between CECs and fibroblasts. All statistical results were verified independently using the Ekuseru-Toukei 2012 (Social Survey Research Information), and the hypergeometric distribution was analyzed using Keisan online calculator (<http://keisan.casio.com>).

ACCESSION NUMBERS

The accession number for the microarray, ATAC-seq and ChIP-seq data reported in this paper is GEO: GSE67823.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.04.020>.

AUTHOR CONTRIBUTIONS

Conceptualization, M.U., S.K., S.K., and S.M.; Investigation, K.K., T.H., T.N., K.M., A.T., T.Y., and S.F.; Formal Analysis, K.K., T.H., M.N., T.Y., A.W., K.O., and S.M.; Resources, M.T. and N.G.; Writing, K.K., S.K., and S.M.

ACKNOWLEDGMENTS

We thank Tetsuro Watabe and Jacob Hanna for critical comments, Takahiro Sato for technical suggestions, and Peter Karagiannis for critical reading of the manuscript. This work was supported in part by CREST, JST (to S.M.), The Japanese Society for the Promotion of Science (JSPS) KAKENHI Grant Number 24615004 (to S.M.) and Number 23390404 (to S.Kinoshita), Core Center for iPS Cell Research, Research Center Network for Realization of Regenerative Medicine from Japan Agency for Medical Research and Development, AMED (to S.M.), iPS Cell Research Fund (to S.M.), and The Funding Program for World-Leading Innovative Research and Development on Science and Technology (FIRST Program) of JSPS (to S.M.).

Received: May 9, 2015

Revised: January 12, 2016

Accepted: March 31, 2016

Published: April 28, 2016

REFERENCES

Buenrostro, J.D., Giresi, P.G., Zaba, L.C., Chang, H.Y., and Greenleaf, W.J. (2013). Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat. Methods* 10, 1213–1218.

Chng, Z., Teo, A., Pedersen, R.A., and Vallier, L. (2010). SIP1 mediates cell-fate decisions between neuroectoderm and mesendoderm in human pluripotent stem cells. *Cell Stem Cell* 6, 59–70.

Conti, L., Pollard, S.M., Gorba, T., Reitano, E., Toselli, M., Biella, G., Sun, Y., Sanzone, S., Ying, Q.L., Cattaneo, E., and Smith, A. (2005). Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS Biol.* 3, e283.

De Craene, B., and Bex, G. (2013). Regulatory networks defining EMT during cancer initiation and progression. *Nat. Rev. Cancer* 13, 97–110.

Di Micco, R., Fontanals-Cirera, B., Low, V., Ntziachristos, P., Yuen, S.K., Lovell, C.D., Dolgalev, I., Yonekubo, Y., Zhang, G., Rusinova, E., et al. (2014). Control of embryonic stem cell identity by BRD4-dependent transcrip-

tional elongation of super-enhancer-associated pluripotency genes. *Cell Rep.* 9, 234–247.

Du, Z.W., Ma, L.X., Phillips, C., and Zhang, S.C. (2013). miR-200 and miR-96 families repress neural induction from human embryonic stem cells. *Development* 140, 2611–2618.

Gill, J.G., Langer, E.M., Lindsley, R.C., Cai, M., Murphy, T.L., Kyba, M., and Murphy, K.M. (2011). Snail and the microRNA-200 family act in opposition to regulate epithelial-to-mesenchymal transition and germ layer fate restriction in differentiating ESCs. *Stem Cells* 29, 764–776.

Graw, J. (2003). The genetic and molecular basis of congenital eye defects. *Nat. Rev. Genet.* 4, 876–888.

Hanna, J., Markoulaki, S., Schorderet, P., Carey, B.W., Beard, C., Wernig, M., Creighton, M.P., Steine, E.J., Cassady, J.P., Foreman, R., et al. (2008). Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. *Cell* 133, 250–264.

Hikichi, T., Matoba, R., Ikeda, T., Watanabe, A., Yamamoto, T., Yoshitake, S., Tamura-Nakano, M., Kimura, T., Kamon, M., Shimura, M., et al. (2013). Transcription factors interfering with dedifferentiation induce cell type-specific transcriptional profiles. *Proc. Natl. Acad. Sci. USA* 110, 6412–6417.

Kao, W.W., Liu, C.Y., Converse, R.L., Shiraishi, A., Kao, C.W., Ishizaki, M., Doetschman, T., and Duffy, J. (1996). Keratin 12-deficient mice have fragile corneal epithelia. *Invest. Ophthalmol. Vis. Sci.* 37, 2572–2584.

Kfoury, Y., and Scadden, D.T. (2015). Mesenchymal cell contributions to the stem cell niche. *Cell Stem Cell* 16, 239–253.

Kitazawa, K., Kawasaki, S., Shinomiya, K., Aoi, K., Matsuda, A., Funaki, T., Yamasaki, K., Nakatsukasa, M., Ebihara, N., Murakami, A., et al. (2013). Establishment of a human corneal epithelial cell line lacking the functional TACSTD2 gene as an in vitro model for gelatinous drop-like dystrophy. *Invest. Ophthalmol. Vis. Sci.* 54, 5701–5711.

Koster, M.I., and Roop, D.R. (2007). Mechanisms regulating epithelial stratification. *Annu. Rev. Cell Dev. Biol.* 23, 93–113.

Lee, T.I., and Young, R.A. (2013). Transcriptional regulation and its misregulation in disease. *Cell* 152, 1237–1251.

Lee, B., Villarreal-Ponce, A., Fallahi, M., Ovadia, J., Sun, P., Yu, Q.C., Ito, S., Sinha, S., Nie, Q., and Dai, X. (2014). Transcriptional mechanisms link epithelial plasticity to adhesion and differentiation of epidermal progenitor cells. *Dev. Cell* 29, 47–58.

Mackay, D.R., Hu, M., Li, B., Rhéaume, C., and Dai, X. (2006). The mouse *Ovol2* gene is required for cranial neural tube development. *Dev. Biol.* 291, 38–52.

Miyoshi, T., Maruhashi, M., Van De Putte, T., Kondoh, H., Huylebroeck, D., and Higashi, Y. (2006). Complementary expression pattern of *Zfx1* genes *Sip1* and *deltaEF1* in the mouse embryo and their genetic interaction revealed by compound mutants. *Dev. Dyn.* 235, 1941–1952.

Ordóñez, P., and Di Girolamo, N. (2012). Limbal epithelial stem cells: role of the niche microenvironment. *Stem Cells* 30, 100–107.

Ouyang, H., Xue, Y., Lin, Y., Zhang, X., Xi, L., Patel, S., Cai, H., Luo, J., Zhang, M., Zhang, M., et al. (2014). WNT7A and PAX6 define corneal epithelium homeostasis and pathogenesis. *Nature* 511, 358–361.

Ozair, M.Z., Kintner, C., and Brivanlou, A.H. (2013). Neural induction and early patterning in vertebrates. *Wiley Interdiscip. Rev. Dev. Biol.* 2, 479–498.

Pellegrini, G., Dellambra, E., Golisano, O., Martinelli, E., Fantozzi, I., Bondanza, S., Ponzin, D., McKeon, F., and De Luca, M. (2001). p63 identifies keratinocyte stem cells. *Proc. Natl. Acad. Sci. USA* 98, 3156–3161.

Perry, K.J., Thomas, A.G., and Henry, J.J. (2013). Expression of pluripotency factors in larval epithelia of the frog *Xenopus*: evidence for the presence of cornea epithelial stem cells. *Dev. Biol.* 374, 281–294.

Schermer, A., Galvin, S., and Sun, T.T. (1986). Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J. Cell Biol.* 103, 49–62.

- Shirakihara, T., Saitoh, M., and Miyazono, K. (2007). Differential regulation of epithelial and mesenchymal markers by deltaEF1 proteins in epithelial mesenchymal transition induced by TGF-beta. *Mol. Biol. Cell* 18, 3533–3544.
- Stergachis, A.B., Neph, S., Reynolds, A., Humbert, R., Miller, B., Paige, S.L., Vernot, B., Cheng, J.B., Thurman, R.E., Sandstrom, R., et al. (2013). Developmental fate and cellular maturity encoded in human regulatory DNA landscapes. *Cell* 154, 888–903.
- Swamynathan, S.K., Katz, J.P., Kaestner, K.H., Ashery-Padan, R., Crawford, M.A., and Piatigorsky, J. (2007). Conditional deletion of the mouse Klf4 gene results in corneal epithelial fragility, stromal edema, and loss of conjunctival goblet cells. *Mol. Cell. Biol.* 27, 182–194.
- Watanabe, K., Villarreal-Ponce, A., Sun, P., Salmans, M.L., Fallahi, M., Andersen, B., and Dai, X. (2014). Mammary morphogenesis and regeneration require the inhibition of EMT at terminal end buds by Ovol2 transcriptional repressor. *Dev. Cell* 29, 59–74.
- Xu, J., Du, Y., and Deng, H. (2015). Direct lineage reprogramming: strategies, mechanisms, and applications. *Cell Stem Cell* 16, 119–134.
- Yamben, I.F., Rachel, R.A., Shatadal, S., Copeland, N.G., Jenkins, N.A., Warmington, S., and Griep, A.E. (2013). Scrib is required for epithelial cell identity and prevents epithelial to mesenchymal transition in the mouse. *Dev. Biol.* 384, 41–52.
- Yoshida, Y., Ban, Y., and Kinoshita, S. (2009). Tight junction transmembrane protein claudin subtype expression and distribution in human corneal and conjunctival epithelium. *Invest. Ophthalmol. Vis. Sci.* 50, 2103–2108.
- Zhang, X., Huang, C.T., Chen, J., Pankratz, M.T., Xi, J., Li, J., Yang, Y., Lavaut, T.M., Li, X.J., Ayala, M., et al. (2010). Pax6 is a human neuroectoderm cell fate determinant. *Cell Stem Cell* 7, 90–100.
- Zhang, T., Zhu, Q., Xie, Z., Chen, Y., Qiao, Y., Li, L., and Jing, N. (2013). The zinc finger transcription factor Ovol2 acts downstream of the bone morphogenetic protein pathway to regulate the cell fate decision between neuroectoderm and mesendoderm. *J. Biol. Chem.* 288, 6166–6177.